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Homology between the *ran1*$^+$ gene of fission yeast and protein kinases

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The *ran1*$^+$ gene of the fission yeast *Schizosaccharomyces pombe* is a negative regulator of both sexual conjugation and meiosis. The nucleotide sequence of the gene has been determined and contains a region of open reading frame (ORF) capable of encoding a protein of 52 000 daltons. S1 nuclease analysis of *ran1*$^+$-encoded RNA showed that the ORF was spanned by an uninterrupted transcript. A fragment of DNA containing the entire *ran1*$^+$ gene was expressed in a bacterial expression vector and found to encode the expected product of 52 000 daltons. The putative *ran1*$^+$ gene product shares significant sequence homology with known protein kinases. The level of the *ran1*$^+$ transcript was similar in vegetative and meiotic cells suggesting that the *ran1*$^+$ protein product rather than its transcript is regulated during sexual differentiation.

Key words: meiosis/*Schizosaccharomyces pombe*/sequence/kinase/*ran1*$^+$

Introduction

The fission yeast, *Schizosaccharomyces pombe*, is a unicellular ascomycete which has a haploid genome during most of its life cycle (Leupold, 1950, 1970). A temporary diploid phase occurs under conditions of nutritional deprivation. Starvation causes cells of opposite mating-type (h^+ and h^-) to undergo sexual conjugation leading to the formation of an h^+/h^- diploid zygote (Egel, 1971). The zygote does not normally propagate vegetatively but instead directly initiates meiosis and sporulation. The ability to undergo meiosis and sporulation is dependent on two conditions: the cell must express both alleles of the mating-type locus (h^+/h^-) and must also be nutritionally starved.

Mutants which bypass both normal meiotic requirements have recently been isolated. They define a single gene known as *ran1*$^+$ (Nurse, 1985) or *pat1*$^+$ (Iino and Yamamoto, 1985a). Here the gene will be referred to as *ran1*$^+$. Strains which carry a temperature-sensitive allele of *ran1* have a complex phenotype. Under conditions which cause a partial loss of *ran1*$^+$ activity, the normal requirement of starvation for the initiation of conjugation and sporulation is bypassed. Thus, if actively dividing *ran1ts* cells are shifted from a permissive to a semi-permissive temperature, they slow their rate of growth, accumulate in the G_1 phase of the cell cycle, conjugate with cells of the opposite mating-type and finally sporulate (Beach *et al.*, 1985; Nurse, 1985).

Complete inactivation of *ran1*$^+$, following transfer of a temperature-sensitive *ran1* mutant from a permissive to a fully non-permissive temperature, has a much more dramatic effect. Vegetative growth and cell division are totally inhibited (Beach *et al.*, 1985) and haploid cells sporulate directly without conjugating (Iino and Yamamoto, 1985a, b; Nurse, 1985; Beach *et al.*, 1985). Both normal requirements for meiosis and sporulation, heterozygosity at the mating-type locus and nutritional starvation, are bypassed following full loss of *ran1*$^+$ activity (Figure 1).

It has been proposed that the *ran1*$^+$ gene is a negative regulator of both conjugation and sporulation (Iino and Yamamoto, 1985a; Nurse, 1985; Beach *et al.*, 1985). The transition from vegetative growth to meiosis is presumed to require gradual inhibition of *ran1*$^+$ activity in two independent steps

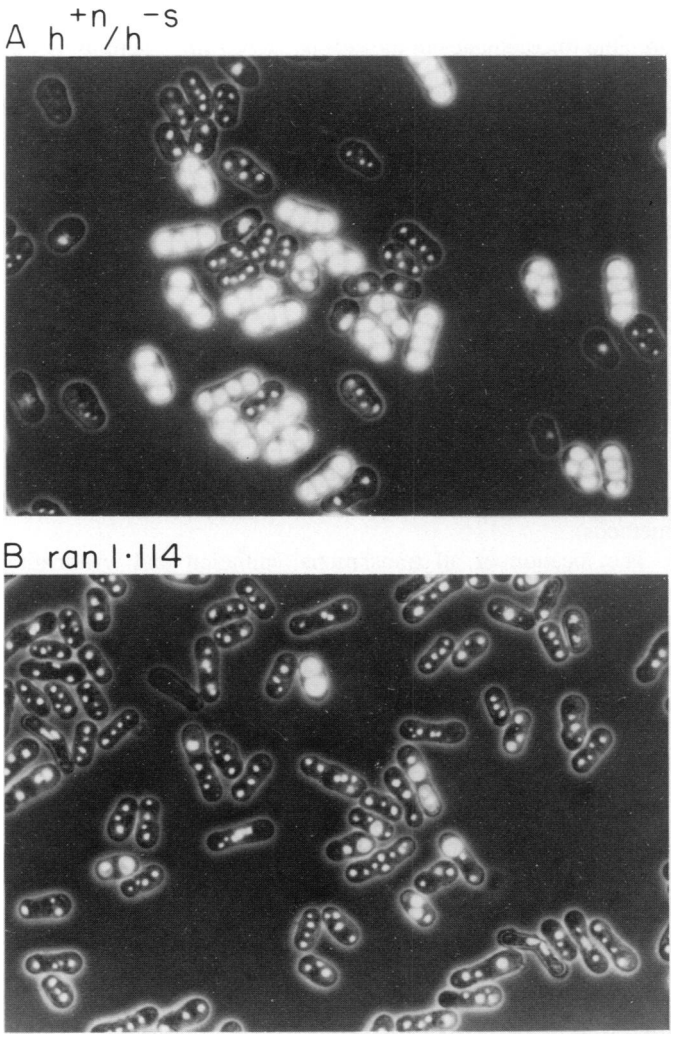

Fig. 1. Meiosis in wild-type and *ran1ts* strains. (**A**) Diploid h^{+N}/h^{-S} strain undergoing meiosis under conditions of nitrogen source starvation. (**B**) Haploid h^{-S} *ran1.114* strain undergoing meiosis and sporulation in complete medium at restrictive temperature (33°C). Cells were fixed in ethanol, treated with RNase and stained with 1 μg/ml propidium iodide. They were photographed under combined phase contrast and fluorescence microscopy allowing visualization of nuclear DNA. Mature spores are impermeable to RNase and therefore stain with propidium iodide with exaggerated intensity.

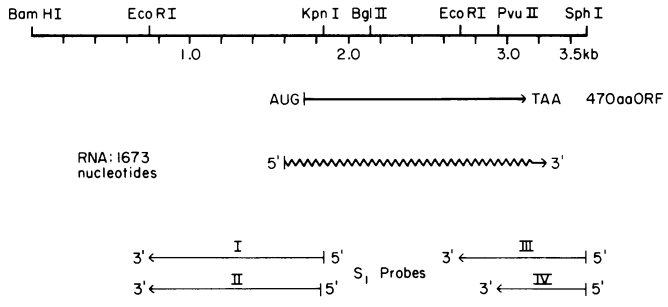

Fig. 2. Restriction map of the 3.5-kb *Bam*HI/*Sph*I fragment containing the *ran1*$^+$ gene. An ORF of 470 amino acids, showing the translational initiation codon (AUG) and the translational termination codon (TAA), is indicated below. The 5′ and 3′ ends of the RNA transcript and the direction of transcription are shown. The probes used for S1 protection studies are also indicated. The precise nucleotide positions of each probe are given in Materials and methods.

(Beach *et al.*, 1985). In the first step, nutritional depletion causes a partial inhibition of *ran1*$^+$ activity and as a result cells of opposite mating-type arrest in the G_1 phase of the cell cycle and are able to conjugate. In the second step, *ran1*$^+$ activity is further inhibited by a mechanism requiring expression of both *matP*$^+$ and *matM*$^+$ mating-type information in the h^+/h^- diploid zygote. Meiosis and sporulation occur once *ran1*$^+$ activity has fallen below a critical threshold.

In order to test this model at the molecular level we have begun a study of the *ran1*$^+$ gene and its protein product. In this paper, we present the nucleotide sequence of the *ran1*$^+$ gene and characterize its transcriptional product.

Results

Nucleotide sequence and transcript map of the ran1$^+$ *gene*

The isolation of the *ran1*$^+$ gene has been described previously (Beach *et al.*, 1985). The nucleotide sequence of a 3.5-kb *Bam*HI/*Sph*I genomic restriction fragment carrying the *ran1*$^+$ gene was determined (Figures 2 and 3, see Materials and methods).

The location of all translational initiation and termination codons was determined and an open reading frame (ORF) which could potentially encode a protein of 470 amino acids was identified. This ORF contains a unique *Bgl*II site (Figure 2) which has previously been shown to be essential for *ran1*$^+$ activity (Beach *et al.*, 1985).

S1 nuclease protection experiments (Berk and Sharp, 1978) were used to determine whether the DNA containing the 470 amino acid ORF is transcribed as RNA. In all such experiments DNA probes uniformly labeled with [^{32}P]dNTP were prepared by primer extension (Burke, 1984) from single-stranded plasmid DNA that contained an appropriate fragment of the *ran1*$^+$ gene (see Materials and methods). The single-stranded probes were hybridized with RNA prepared from a wild-type yeast strain, digested with S1 nuclease and analyzed by gel electrophoresis. A DNA probe extending from the *Kpn*I site (position 1836, Figure 2) to the *Eco*RI site (position 2750, Figure 2) was fully protected from S1 nuclease digestion (data not shown). This probe is contained completely within the ORF. Probe I, which spans the 5′ end of the gene (Figure 2) yields a major protected doublet of approximately 240 bases (Figure 4a, lane 4, 5). In certain experiments, a high mol. wt band was observed which is most likely the result of incomplete nuclease S1 digestion. In order to determine if the protected doublet represents the 5′ initiating site of the transcript or a splice junction, a second probe was used. Probe

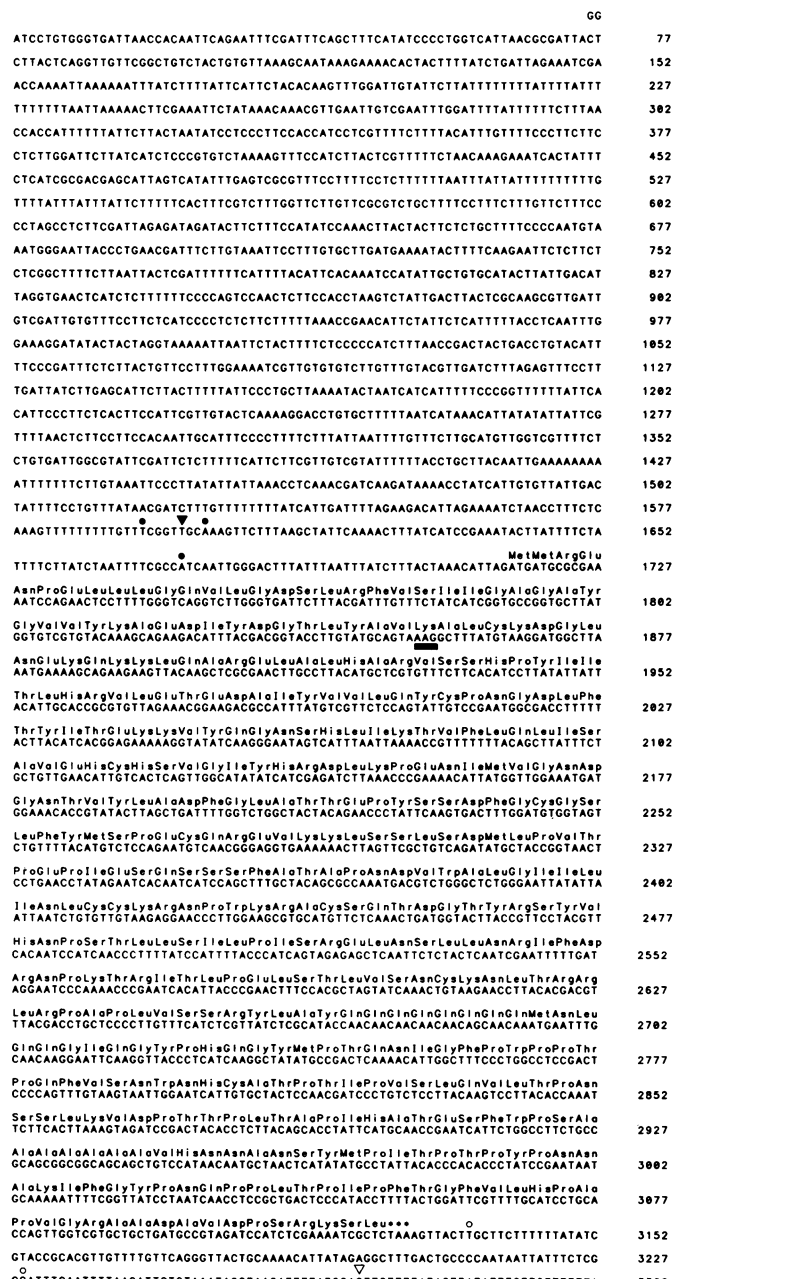

Fig. 3. Nucleotide sequence of *ran1*$^+$. Only the *ran1*$^+$ coding strand is shown. Nucleotide +1 is defined by a unique *Bam*HI site. The major transcriptional start site is highlighted by a filled arrow and the minor sites by filled circles. The major 3′ end of the transcript is represented by an open arrow and the minor stop sites by open circles. The proposed amino acid sequence is illustrated. The lysine corresponding to Lys-71 of cAMP-dependent kinase is indicated by a filled box.

II (Figure 2) is identical to probe I except that probe I extended 38 bases further than probe II into 470 amino acid ORF. A major protected band of 205 nucleotides was detected following hybridization with DNA probe II (Figure 4A lane 3). Since the length of the protected bands observed with probes I and II differ by 38 nucleotides, the transcript must span the 5′ end of both probes. The length of the 5′ protected fragments indicates that there is a discontinuity in the DNA/RNA hybrid which maps to nucleotide position 1599, 243 bp from the *Kpn*I site (Figures 2

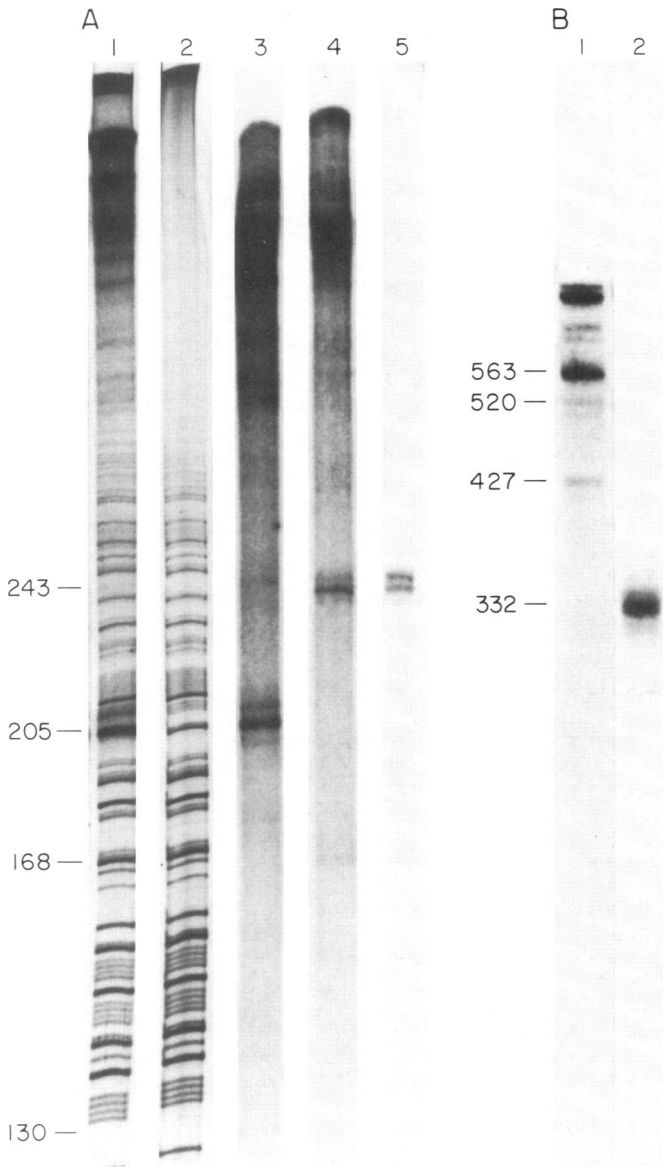

Fig. 4. S_1 mapping of the *ran1*$^+$ transcript. **(A) Lane 1:** Mixed loading of the material applied to both lanes 2 and 3. **Lane 2:** DNA sequencing ladder (A-lane) primed with the same oligonucleotide used to synthesize probe II (see Materials and methods). **Lane 3:** fragment of probe II protected from S1 nuclease after hybridization with yeast RNA. The length of the protected fragment (205 base) could be precisely determined from the sequencing ladder of the corresponding DNA (lanes 1,2). **Lane 4:** fragment of probe I protected from S1 nuclease after hybridization with yeast RNA. The major protected band is 243 bp in length, 38 bp longer than the band obtained with probe I. **Lane 5:** fragment of probe I protected from S1 nuclease. The length of this fragment was estimated to be 240 bases by comparison with ^{32}P- labeled M13Mp18 digested with *Hpa*I. **(B)** Fragment of probe III (**lane 1**) or probe IV (**lane 2**) protected from S1 by hybridization with yeast RNA. The mol. wt of the bands was estimated by comparison with ^{32}P-labeled M13Mp18 digested with *Hpa*I.

and 3). Since this site does not contain sequences normally associated with the 3′ end of a pre-mRNA splice junction (Breathnach *et al.*, 1978), it is likely that nucleotide position 1599 (Figure 3) marks the 5′ end of the RNA transcript. This site is 116 bp from the first AUG of the 470 amino acid ORF. No other AUG codons, in any of the three possible reading frames, lies between the 5′ end of the transcript and the first methionine codon of the long ORF. Minor transcriptional initiation points were also observed at nucleotide positions 1602 and 1674 (Figures 3 and 4).

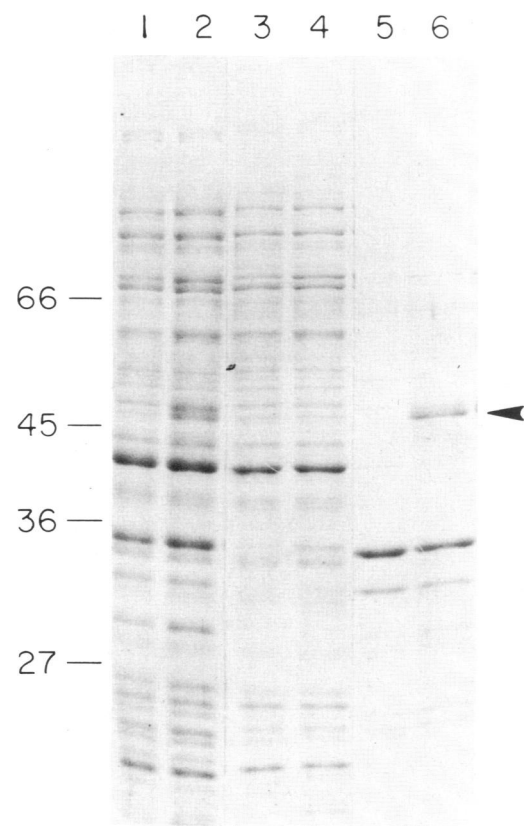

Fig. 5. Expression of the 52-kd *ran1*$^+$ gene product in *E. coli*. Proteins of *E. coli* strain BL21 (DE3) lysS carrying pRan1.38 before or after induction with IPTG. **Lanes 1** and **2**: total cell lysate from 0.2 ml cells without induction (lane 1) or 2 h after induction (lane 2). **Lanes 3** and **4:** soluble fraction of uninduced (lane 3) or induced (lane 4) cell lysates. **Lanes 5** and **6:** insoluble fraction of uninduced (lane 5) or induced (lane 6) cell lysates.

The 3′ end of the transcript was mapped using two probes which were identical except that probe III extended 231 nucleotides further into the region of ORF than probe IV (Figure 2). Probes III and IV yielded major protected fragments of ~563 nucleotides and 332 nucleotides respectively (Figure 4B). These data suggest that the 3′ end of the transcript lies at approximately nucleotide position 3272 (Figure 3), 144 bp from the termination codon of the 470 amino acid ORF. Minor protected bands were observed with probes III and IV and suggest the existence of multiple 3′ end-points occurring at nucleotide positions 3329 and 3126 (Figures 3 and 4).

The 5′ end of a transcript has been mapped 116 bp upstream of the initiating AUG of the 470 amino acid ORF and its 3′ end has been mapped 144 bp downstream of the terminating TAA codon. Since the 470 amino acid ORF contains the *Bgl*II site which is known to be essential for *ran1*$^+$ activity (Beach *et al.*, 1985) and since the entire ORF is spanned by an uninterrupted RNA transcript, we conclude that the primary translational product of the *ran1*$^+$ is likely to be a protein of 470 amino acids with a calculated mo. wt of 52 167 daltons.

Expression of the ran1$^+$ *protein in Escherichia coli*

The *ran1*$^+$ protein was obtained in high amounts using the T7 polymerase gene expression system (Studier and Moffatt, 1986; see Materials and methods). In this system, the gene for T7 RNA polymerase is integrated into the *E. coli* chromosome under the control of the inducible *lac* UV5 promoter and the gene to be expressed is cloned onto a modified pBR322 plasmid under the

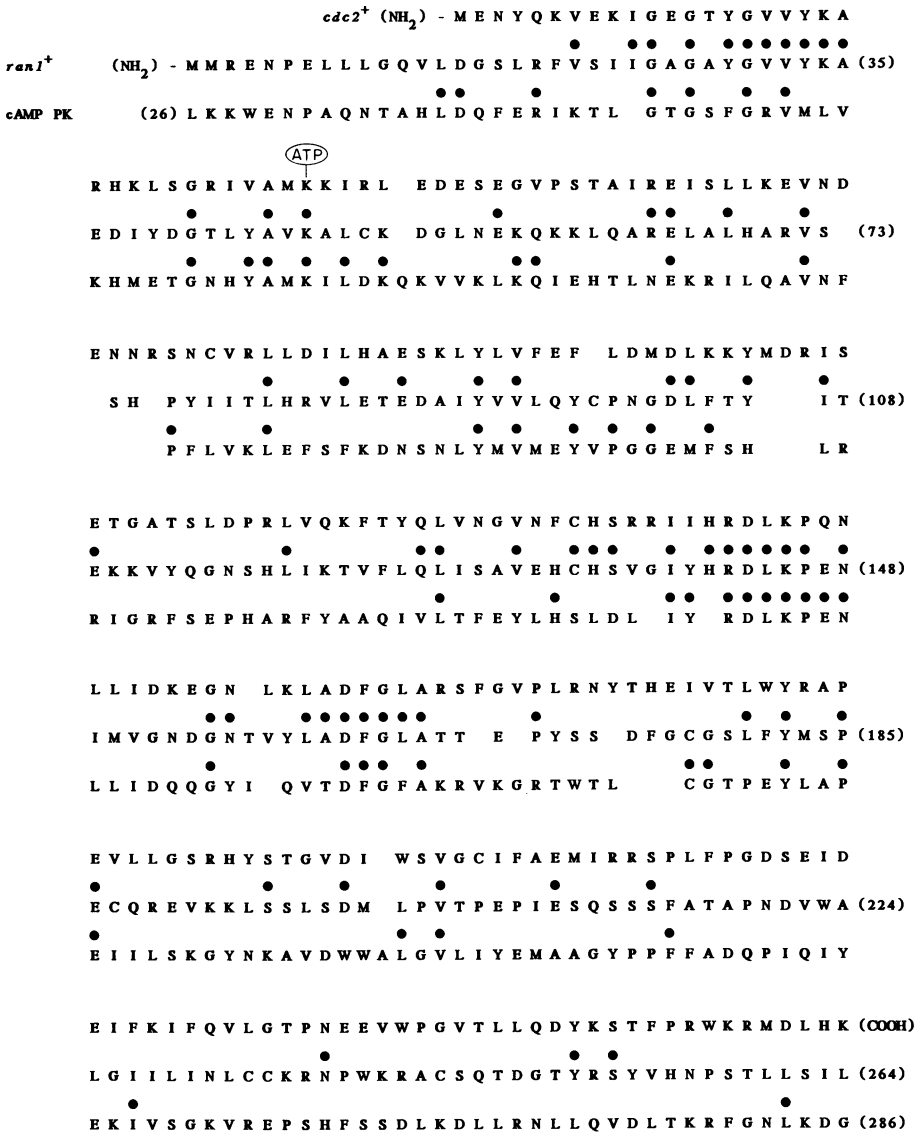

Fig. 6. Homology between *ran1*$^+$, the *cdc2*$^+$ gene of fission yeast and bovine cAMP-dependent protein kinase. **Top line:** *cdc2*$^+$ (amino acids 1–297; taken from Hindley and Phear, 1984). **Middle line:** *ran1*$^+$ (amino acids 1–264). **Bottom line:** bovine cAMP-dependent protein kinase (amino acids 26–327; taken from Shoji *et al.*, 1981). The amino acid sequences have been aligned to give maximum homology. The site of ATP binding in cAMP-dependent protein kinase is indicated.

control of the T7 gene 10 promoter (Studier and Moffatt, 1986; Studier, unpublished).

A fragment of *ran1*$^+$ DNA was modified by insertion of an *Nde*I site at the initiating ATG in order to allow cloning of the gene into the *Nde*I site present in the T7 expression vector pAR3038 (Studier, unpublished) to form pRan1.38. Therefore, pRan1.38 contains the entire *ran1*$^+$ gene including the initiating methionine under the control of a T7 promoter. Following introduction of this plasmid into BL21(DE3)LysS (Studier, unpublished) and induction of the T7 polymerase with isopropyl-β-D-thiogalactopyranoside, the plasmid directed synthesis of a 52 000-dalton protein (Figure 5, lanes 2 and 6). The *ran1*$^+$ protein is found in the insoluble fraction of the cell lysate. The production of a 52 000-dalton protein confirms that the *ran1*$^+$ gene does indeed contain the predicted region of the ORF. *ran1*$^+$ protein expressed in *E. coli* will be used for the generation of anti-*ran1*$^+$ antibodies.

Homology between ran1$^+$ *product and protein kinases*

The sequence of the 52 000-kd *ran1*$^+$ gene product was used in a computer search of the Protein Identification Resource (NBRF) and GenBank protein data bases. Significant homology was found between *ran1*$^+$ and the entire family of protein kinases. The greatest degree of homology was observed between *ran1*$^+$ and either bovine cAMP-dependent protein kinase or the *cdc2*$^+$ cell cycle control gene of fission yeast (Figure 6). The product of *cdc2*$^+$ has recently been shown to be a protein kinase (Simanis and Nurse, 1986).

The homology between the predicted *ran1*$^+$ gene product and 3′5′ cAMP-dependent protein kinase is restricted to an ~30-kd region which is the most highly conserved among protein kinases and contains the catalytic domain (Hunter and Cooper, 1985). Most conspicuously, the *ran1*$^+$ protein has a lysine residue at position 47 which can be precisely aligned with Lys 71 of cAMP-dependent protein kinase (Shoji *et al.*, 1981). Lys 71 is the residue identified as the site of ATP binding (Zoller *et al.*, 1981). Lys 71 lies 20 residues from the highly conserved sequence Gly-N-Gly-N-N-Gly. which has been found in all protein kinases (Hunter and Cooper, 1985) and is present in *ran1*$^+$. Towards the car-

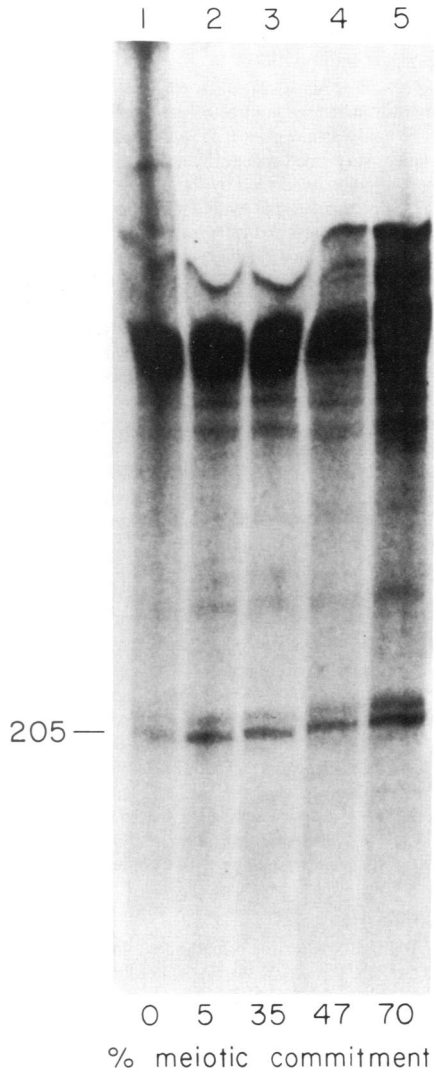

Fig. 7. Level of *ran1*$^+$ transcript during meiotic initiation in an h^+/h^- strain. The figures beneath each lane indicate the percentage of the culture which was irreversibly committed to meiosis at the time of harvesting for RNA preparation. The means by which this percentage was estimated is described in Materials and methods. Probe II (Figure 2) was annealed with 20 μg whole cell RNA, treated with S1 nuclease and visualized following 6% acrylamide gel electrophoresis.

boxyl terminus of the molecule, a second functionally important region has been identified which is also highly conserved among all protein kinases. In the *ran1*$^+$ protein this region corresponds to codons 142−186. These homologies suggest that *ran1*$^+$ is a protein kinase.

The level of the ran1$^+$ *transcript does not decrease during meiosis*

In the budding yeast, *Saccharomyces cerevisae*, transcriptional control of cell type-specific genes by the mating-type locus is well documented (Jensen *et al.*, 1983). For example, the transcriptional activity of the *RME* meiotic control gene is inhibited in a/α diploid cells (Mitchell and Herskowitz, 1986). It was expected that the mating-type genes of fission yeast might control the level of *ran1*$^+$ transcription.

The *ran1*$^+$ transcript was examined in h^+/h^- diploid cells entering meiosis. The abundance of the transcript did not decline during meiosis. Instead a small but consistent increase was observed (Figure 7). In addition, the transcript was found at very similar levels in vegetatively growing haploid cells (data not shown). These data suggest that transcriptional regulation plays little if any role in the control of the activity of *ran1*$^+$.

Discussion

We have presented the nucleotide sequence of the *ran1*$^+$ gene and show that it contains an ORF of 470 amino acids. The gene generates a 1700-nucleotide transcript containing 5′ and 3′ untranslated regions of 116 and 144 nucleotides, respectively. The abundance of the transcript is similar both in vegetative growth and during entry into meiosis. The anticipated product of the *ran1*$^+$ gene shares homology with the family of protein kinases.

These observations suggest that the *ran1*$^+$ product is probably a protein kinase. This is of particular interest because it has previously been shown that expression of the *ran1ts* phenotype is suppressed under conditions in which 3′5′ cAMP-dependent protein kinase is abnormally active (Beach *et al.*, 1985). This implies that there is some overlap in the function of cAMP-dependent protein kinase and the product of the *ran1*$^+$ gene. The degree of homology between bovine cAMP-dependent protein kinase and the *ran1*$^+$ gene is significant but does not necessarily indicate that the *ran1*$^+$ product is itself a cAMP-dependent kinase. Rather, we suppose that *ran1*$^+$ is a related protein kinase which may share certain key substrates with cAMP-dependent protein kinase.

Inhibition of *ran1*$^+$ activity has been predicted to be an essential step in meiotic initiation. If inhibition of *ran1*$^+$ is essential, the mating-type genes might be expected to control *ran1*$^+$ activity. In *S. cerevisiae*, the mating-type genes are DNA binding proteins which control the expression of other genes (Johnson and Herskowitz, 1985). We have shown that the level of the *ran1*$^+$ transcript is not regulated by the mating-type genes since it is equally abundant during both vegetative growth and meiosis. The mating-type genes might regulate *ran1*$^+$ activity indirectly, by controlling the expression of one or more intermediate genes, which in turn directly interact with the *ran1*$^+$ gene product. One such gene may be *mei3*$^+$ which is required specifically for meiotic initiation (Bresch *et al.*, 1968). The *mei3*$^+$ gene is expressed only during meiosis. If, however, *mei3*$^+$ is expressed in vegetative cells, growth is inhibited and haploid meiosis and sporulation occur (Beach and Stein, in preparation). This phenotype is identical to that of a recessive *ran1* mutant, suggesting that *mei3*$^+$, expressed under mat control, acts to inhibit *ran1*$^+$.

There are numerous ways in which *mei3*$^+$ might regulate *ran1*$^+$. The *mei3*$^+$ product might act in the modification or maturation of the *ran1*$^+$ product or serve as a regulatory subunit. Alternatively, *mei3*$^+$ could control the expression of other, still unidentified meiotic genes, whose products interact with the *ran1*$^+$ protein. Investigation of the mechanism of regulation of *ran1*$^+$ and characterization of its biochemical properties will be possible once antibodies have been raised against the protein.

Materials and methods

Nucleotide sequencing

In order to sequence the 3.5-kb *Bam*HI/*Sph*I restriction fragment containing *ran1*$^+$ fragment, four subclones were obtained in M13Mp18 (Yanish-Perron *et al.*, 1985). pM30 and pM31 contained the 1.8-kb *Bam*HI/*Kpn*I fragment in opposite orientations; pM32 and pM33 contain the 1.7-kb *Kpn*I/*Sph*I fragment in opposite orientations. In each of these constructs, the *ran1*$^+$ fragment was blunt-ended (Henikoff, 1984) and cloned into the *Sma*I site of the phage vector. The plasmids were used to create unidirectional deletions in the *ran1*$^+$ fragment by the method of Henikoff (1984). Briefly, the replicative form of the recombinant

phage was obtained and linearized using *Bam*HI and *Sph*I (New England Biolabs) both of which cut in the polylinker. The linear DNA was treated with ExonucleaseIII (New England Biolabs) for increasing intervals of time. S1 nuclease and Klenow polymerase (Bethesda Research Laboratories) were used to create blunt ends and after ligation the DNA was transformed into *E. coli* (TG1). The extent of the deletion into the *ran1*$^+$ fragment varied with time of exposure to ExonucleaseIII. Single-strand phage was isolated and used as a template for sequencing reactions. Chain termination sequencing reactions using α[^{35}S]dATP (New England Nuclear) were performed as described in Sanger *et al.* (1980) and Biggin *et al.* (1983) using universal primer. The products of sequencing reactions were resolved on 6% polyacrylamide gels (Maxam and Gilbert, 1980). Fully overlapping DNA sequence in both orientations was obtained.

S1 mapping

Single-stranded probes were prepared by cloning the appropriate fragment (see Figure 2 and text) into pUC118 (Vieira, personal communication). Probes I and II contain the 1.1-kb *Eco*RI/*Kpn*I fragment. Probe III contains the 785-bp *Eco*RI/*Sph*I fragment and probe IV contains the 554-kb *Pvu*I/*Sph*I fragment. pUC118 contains the intragenic region of M13 phage and is secreted as single-strand phage following superinfection of the defective phage M13K07 (Vieira, personal communication). Single-strand plasmid DNA was isolated and annealed with either universal primer (probes I, III, IV) or the 17-mer 5′ CATAAGCACCGGCACCG 3′ (nucleotides 1787–1803; probe II) to synthesize a radioactively labeled second strand (Burke, 1984). The DNA was digested with *Eco*RI and following denaturation the labeled single-stranded fragment was isolated on 4% polyacrylamide–urea gels. The fragment was purified from the gel by electroelution into dialysis bags. Total RNA was isolated from yeast cells by a modification of the method of Beggs *et al.* (1980) and 20 μg was hybridized with DNA probe in 0.25 M NaCl, 0.032 M Hepes pH 7.6, 3 mM EDTA. Hybridization reactions were incubated at 65°C for 18 h. The hybrids were digested with 100 units S1 (B.R.L.) in 4 mM $ZnSO_4$, 30 mM NaOAc pH 4.6, 0.25 M NaCl and the protected fragments were resolved on either 4% or 6% polyacrylamide gels.

Preparation of whole cell RNA

RNA was prepared from *S. pombe* strain 972 (h^{-S}) cultured at 30°C in 250 ml minimal media (Mitchison, 1970). Cells were harvested during exponential growth, chilled on ice, and washed in cold water. Sterile glass beads (10 ml, 0.45 μm) were added to the pelleted cells and cold breaking buffer (0.32 M sucrose, 20 mM Tris–HCl pH 7.5, 10 mM EDTA, 0.5 mg/ml Heparin) was added until the meniscus just covered the glass beads. The cells were broken by vortexing for 1 min. Dilution buffer (20 ml; 50 mM Tris–HCl pH 7.5, 100 mM NaCl, 5 mM EDTA, 1% SDS, 0.5 mg/ml Heparin) was added to the broken cells along with an equal volume of phenol. The mixture was vortexed vigorously and the aqueous phase isolated. The aqueous phase was extracted twice more with phenol and finally with phenol/chloroform. The RNA was precipitated from the aqueous solution following addition of LiCl to 0.5 M and ethanol to 70%.

Assay of meiotic commitment

A diploid h^{-S}*ade6.210*/h^{+N}*ade6.216* strain was cultured to stationary phase in minimal media containing 3% glucose and 100 mM NH_4Cl. Meiosis is not initiated in this medium even in cells entering stationary phase. Stationary cells were washed and incubated in minimal media containing no nitrogen source and 0.1% glucose, 1% glycerol as a carbon source. Meiosis is rapidly initiated with relatively little increase in cell number (<50%) under these conditions. RNA was prepared from fractions of the culture at time intervals (1, 2.5, 4 and 6 h) after incubation in the glycerol/glucose medium. The percentage of cells committed to meiosis was assayed at the same time intervals. The h^{+N}*ade6.219*/h^{-S}*ade6.216* diploid is phenotypically *Ade*$^+$ due to intragenic complementation of the *ade6.210* and *ade6.216* alleles. Cells taken from the sporulating culture were plated on complete media containing 10 μg/ml adenine, which supports growth of *Ade*$^-$ colonies but causes them to turn pink, whereas *Ade*$^+$ colonies are white. Diploids committed to the completion of meiosis and sporulation at the time of plating segregate *ade6.210* and *ade6.216* spores which germinate and form pink colonies. Diploids not committed to meiosis at the time of plating form colonies which contain only *ade6.210*/*ade6.216* diploid cells and therefore remain white. The ratio of pink:total colonies, at each interval after glucose/glycerol addition, gives an estimate of meiotic commitment.

Expression of ran1$^+$ *in E. coli*

Plasmids. The plasmid pAR3038 consists of pBR322 with a 118-nucleotide fragment containing the promoter of the T7 gene 10 in the *Bam*HI site. The promoter is adjacent to a unique *Nde*I site suitable for insertion of target genes (F.W.Studier, unpublished). An *Nde*I site was created at the initiating methionine of the *ran1*$^+$ gene by oligonucleotide mutagenesis in the following manner: pRan1.35 consists of pUC118 carrying the entire *ran1* *Bam*HI/*Sph*I fragment (see Figure 2). Single-stranded phage DNA was obtained from this plasmid and annealed with the 24-mer 5′ TTCGCGCATCATATGTTTACTAAA 3′. Mutagenesis was as described (Zoller and Smith, 1984) and plasmid pRan1.36 was obtained which is identical to pRan1.35 except that it contains an *Nde*I site (5′ CATATG 3′) at the initiating methionine of the gene. *ran1*$^+$ was cloned as a *Nde*I/*Hin*dIII fragment into pAR3038.

Expression. The *E. coli* strain BL21(DE3) contains a chromosomal copy of the T7 RNA polymerase under the control of the *lac* UV5 promoter (Studier and Moffatt, 1986). When plasmid pRan1.38 was used to transform this strain to ampicillin resistance, very small transformants were obtained. They never grew to the normal size of colonies formed by the same strain harboring the plasmid pAR3038. It has been reported that even under non-inducing conditions, a low level of T7 polymerase is present and can prevent the establishment or maintenance of plasmids carrying toxic genes (Studier and Moffatt, 1986). In order to overcome this problem, strain BL21(DE3) LysS was used (F.W.Studier, unpublished). This strain is BL21 (DE3) carrying the gene for T7 lysozyme on the plasmid pAC184. T7 lysozyme binds to the T7 RNA polymerase and thus the basal level of polymerase is lowered until it is strongly induced with IPTG. The plasmid also confers on the cell chloramphenicol resistance and is compatible with pBR322-based plasmids.

pRan1.38 was introduced into BL21(DE3) LysS and rapidly growing ampicillin/chloramphenicol-resistant colonies were obtained without difficulty. Individual transformants were grown at 37°C in 10 ml L broth (Miller, 1972) containing 50 μg/ml ampicillin and 10 μg/ml chloramphenicol. When the culture reached OD_{600} 0.2, glycerol was added to a final concentration of 15% and the cells were frozen at −70°C. For production of the *ran1*$^+$ protein frozen cells were thawed and 10 μl λ inoculated into 10 ml L broth containing ampicillin and chloramphenicol. Cultures were induced with 0.4 mM IPTG (Sigma) when they reached OD_{600} 0.6 and cells were harvested by centrifugation 2 h after induction. Cell pellets were stored frozen at −70°C.

Preparation of cell lysates. Frozen pellets (10 ml cells) were thawed on ice and resuspended in 1.0 ml 50 mM Tris–HCl pH 7.8, 2 mM DTT, 5 mM EDTA, 2 mM benzamadine HCl, 1 mM PMSF, 10% glycerol, 0.3% Triton X-100. After incubation on ice for 10 min, cells were sonicated four times for 10 s. The extract was clarified by centrifugation at 10 000 *g* for 10 min. Insoluble material was solubilized in sample buffer (Laemmli, 1970) before gel electrophoresis. Total cell extract was prepared by resuspending the frozen cell pellets in sample buffer (Laemmli, 1970). *E. coli* proteins were separated on 12% polyacrylamide gels and proteins were visualized by staining with Coomassie Brilliant Blue (Sigma).

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References

Beach,D., Rodgers,L. and Gould,J. (1985) *Current Genet.*, **10**, 297–311.
Beggs,J.D., van den Berg,J., van Ooyen,A. and Weissman,C. (1980) *Nature*, **283**, 835–840.
Berk,A.J. and Sharp,P.A. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 1274–1278.
Biggin,M.D., Gibson,T.J. and Hong,G.F. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3963–3965.
Breathnach,R., Benoise,C., O'Hare,K., Gannon,F. and Chambon,P. (1978) *Proc. Natl. Acad. Sci. USA*, **75**, 4853–4857.
Bresch,C., Muller,G. and Egel,R. (1968) *Mol. Gen. Genet.*, **102**, 301–306.
Burke,J.F. (1984) *Gene*, **30**, 63–68.
Egel,R. (1971) *Planta*, **98**, 89–96.
Henikoff,S. (1984) *Gene*, **28**, 351–359.
Hindley,J. and Phear,G. (1984) *Gene*, **31**, 129–134.
Hunter,T. and Cooper,J.A. (1985) *Annu. Rev. Biochem.*, **54**, 897–930.
Iino,Y. and Yamamoto,M. (1985a) *Mol. Gen. Genet.*, **198**, 416–421.
Iino,Y. and Yamamoto,M. (1985b) *Proc. Natl. Acad. Sci. USA*, **82**, 2447–2451.
Jensen,R., Sprague,G.F.Jr and Herskowitz,I. (1983) *Proc. Natl. Acad. Sci. USA*, **80**, 3035–3039.
Johnson,A.D. and Herskowitz,I. (1985) *Cell*, **42**, 237–247.
Laemmli,U.K. (1970) *Nature*, **227**, 680–685.
Leupold,U. (1950) *C.R. Trav. Lab. Carlsberg Ser. Physiol.*, **24**, 381–480.
Leupold,U.(1970) In Prescott,D.M. (ed.), *Methods in Cell Physiology*. Academic Press, New York, pp. 169–177.
Maxam,A.B., and Gilbert,W. (1980) *Methods Enzymol.*, **65**, 499–560.
Miller,J.H. (ed.) (1972) *Experiments in Molecular Genetics*. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
Mitchell,A.P. and Herskowitz,I. (1986) *Nature*, **319**, 738–742.

Mitchison,J.M. (1970) In Prescott,D.M. (ed.), *Methods in Cell Physiology*. Academic Press, New York, Vol. 4.
Nurse,P.M. (1985) *Mol. Gen. Genet.*, **198**, 497–502.
Sanger,F., Nicklen,S. and Coulson,A.R. (1977) *Proc. Natl. Acad. Sci. USA*, **74**, 5463–5467.
Shoji,S., Parmelee,D.C., Wade,R.D., Ericsson,L.H., Walsh,K.A., Neurath,H., Long,G.L., Demaille,J.G., Fischer,E.H. and Titani,K. (1981) *Proc. Natl. Acad. Sci. USA*, **78**, 848–851.
Simanis,V. and Nurse,P.M. (1986) *Cell*, **45**, 261–268.
Studier,F.W. and Moffatt,B.A. (1986) *J. Mol. Biol.*, **189**, 113–130.
Yanish-Perron,C., Vieira,J. and Messing,J. (1985) *Gene*, **33**, 103–109.
Zoller,M.J. and Smith,M. (1984) *Gene*, **3**, 479–488.
Zoller,M.J., Nelson,N.C. and Taylor,S.S. (1981) *J. Biol. Chem.*, **256**, 589–592.

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